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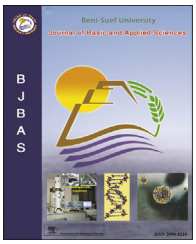

Full Length Article

Limited transmission of multidrug-resistant tuberculosis in East Azarbaijan, Iran

Mohammad Asgharzadeh [a], *Hossein Samadi Kafil* [b,*], *Mahya Pourostadi* [c]

[a] *Biotechnology Research Center, Faculty of Paramedicine, Tabriz University of Medical Sciences, Tabriz, Iran*

[b] *Drug Applied Research Center, Faculty of Medical Sciences, Tabriz University of Medical Sciences, 5166614766 Tabriz, Iran*

[c] *Tuberculosis and Lung Disease Research Center, Tabriz University of Medical Sciences, Tabriz, Iran*

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ABSTRACT

Tuberculosis is the leading cause of death in adults from a single infectious agent, killing about 3 million people every year. One-third of the human population is thought to be infected by the causative agent *Mycobacterium tuberculosis*. Multidrug resistance isolates are one of the most important concerns in tuberculosis control. In this study, we used MIRU-VNTR to determine MDR-TB transmission in Tabriz. A hundred and twenty *M. tuberculosis* isolates were collected from patients who referred to TB center of Tabriz and characterized for drug resistance and typing by MIRU-VNTR method. Thirty percent of the isolates were resistant to at least one drug. Among Iranian isolates only two isolates (1.9%) were MDR and in patients from the Republic of Azerbaijan four isolates (28.6%) were MDR. By MIRU-VNTR typing eighty eight distinct profiles were identified, including 20 clustered profiles and 68 unique patterns and fifty two isolates (43.3%) included in clusters. The minimum estimate of the proportion of tuberculosis transmission in East Azerbaijan with MIRU-VNTR was 26.7% and 14% of MDR isolates acquired infections recently. Results of the present study showed that high percent of TB cases are sensitive to antibiotics. MIRU-VNTR typing showed a limited number of MDR-TB transmission in our isolates set. Because of the increasing number of patients referred from Rep. of Azerbaijan with MDR-TB, we need more stewardship and controlling programs for these patients for preventing the outbreak of MDR-TB in our region.

1. Introduction

The importance of the tuberculosis (TB) a major infectious disease and a cause of high morbidity and mortality has been completely recognized (Asgharzadeh et al., 2011). Countless millions of people have died from tuberculosis, a chronic infectious disease caused by the tubercle bacillus (Cole et al., 1998). The World Health Organization (WHO) estimated that there were about 450,000 new multidrug-resistance (MDR)-TB

* *Corresponding author*. Tel.: +98 9127184735.
E-mail address: Kafilhs@tbzmed.ac.ir (H.S. Kafil).

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cases in the world in 2012. About 170,000 MDR-TB deaths were estimated to have occurred in 2012 (WHO, 2013). About 10% of MDR-TB cases were XDR-TB (Extensively drug-resistant-TB). By September 2013, 92 countries had reported at least one XDR-TB cases [3]. MDR-TB is caused by resistance to isoniazid (INH) and rifampin (RF) (Mokrousov et al., 2003) and extensively drug-resistant (XDR)-TB is caused by *Mycobacterium tuberculosis* isolates resistant to rifampin and isoniasid and fluoroquinolone, and one of the three injectable drugs, capreomycin, kanamycin and amikacin (Gandhi et al., 2010a). MDR-TB is a problem both in the developed as well as in the developing countries. The problem is even more serious among the human immunodeficiency virus (HIV) infected population (Gandhi et al., 2010b; Wells et al., 2007). Although treatment of MDR is possible with currently available diagnostic techniques and drugs, the treatment course is substantially more close and laborious than from drug susceptible tuberculosis, with higher rates of treatment failure and mortality (Gandhi et al., 2010a).

Mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) is an invaluable tool for genotyping MIRU-VNTR revealed more transmission and clusters (Asgharzadeh et al., 2008; Mazars et al., 2001).

The province of East Azarbaijan is located in North West of Iran and neighbor with republic of Azarbaijan (Fig. 1). The estimated population of the province is approximately 3,730,000 of which about 40% are inhabitants of Tabriz, the capital city of the province.

Based on the report of WHO, the Republic of Azerbaijan is one of high TB and MDR-TB burden countries (WHO, 2013), and Azeri patients travel to Tabriz for treatment of TB. In Tabriz, the TB surveillance is based on conventional methods which can't identify the source of infection and discriminate between new and old infections. Thereby, in this study, we used MIRU-VNTR to determine MDR-TB transmission in Tabriz.

2. Material and methods

2.1. *Patients population and bacterial isolates*

All isolates of *M. tuberculosis* were collected from patients who referred to TB center of Tabriz. The study population comprised all patients from whom at least one sample was positive for *M. tuberculosis* by culture. 120 isolates of *M.*

Fig. 1 – East Azarbaijan province of Iran in neighborhood of Rep. Azerbaijan, Armenia, Turkey and Iraq.

tuberculosis were collected. 14 patients were from the Republic of Azerbaijan and 106 patients were from East Azerbaijan of Iran.

Clinical isolates were recovered from sputum ($N = 92$), bronchial fluids ($N = 19$), Cerebrospinal fluids ($N = 2$), Urine ($N = 2$), neck mass biopsy ($N = 1$), endometrial biopsy ($N = 1$) and pleural fluid ($N = 1$). All isolates were cultured in lowenstein jensen medium. The species identification of the isolates was based on standard biochemical tests, including production of niacin, catalase activity, nitrute reduction, and pigment production and growth rate. The susceptibilities of isolates to INH, RF, Streptomycin (SM), and ethambutol (ETB) were determined by the proportion method (Rieder et al., 1998). Information about age, sex, geographical origin and history of tuberculosis were collected by the staffs of TB center.

2.2. DNA extraction

DNA used for the PCR analysis was extracted from cultured *M. tuberculosis*. Two loops full of bacteria were suspended in 400 μl of TE buffer (10 mM Tris-cl, 1 mM EDTA, pH 8.0) and placed at 80 °C for 20 min to kill the bacteria. DNA was extracted by lysozyme, SDS, proteinase K, NaCl, CTAB and Chloroform–isoamylalcohol. Extracted DNA after sedimentation with isopropanol and washing with ethanol 70% was redissolved in 50 μl of dionized water and stored at −20 °C (Asgharzadeh et al., 2007).

2.3. MIRU-VNTR

PCR was performed in 20 μl volume that contained 5–50 ng of DNA, 0.5 μM of specific primers (Supply et al., 2001) in the presence of 1.5 mM $MgCl_2$, 100 μM of each dNTP, 50 mM KCl, 20 mM Tris-CL, pH 8.4, and 1.25 U recombinant Taq DNA polymerase (Cinnagen, Iran). All PCRs were initiated by a 7 min denaturation step at 94 °C and completed by a 7 min extension step at 72 °C. The temperature cycles for different types of PCRs were as follows, 35 cycles of 45 s at 94 °C, annealing temperature for 45 s and final 72 °C for 55 s. Annealing temperature were used as follows: 65,63, 68, 65, 59, 67, 59, 65, 64, 63, 68 and 65 for MIRU loci 2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39 and 40 respectively (Supply et al., 2001). Negative controls consisted of the PCR components of reaction mixture lacking mycobacterial DNA. PCR products were electrophoresis in 1.5% agarose gel and after staining with 0.5 μg/ml ethidium bromide in comparing with 100 bp-plus DNA ladders size marker (Fermentas, Lithuania). Reference strain *M. tuberculosis14323* was used as standard strain for PCR. MIRU-VNTR plus website was used for analyzing MIRU-VNTR results (www.miru-vntrplus.org/).

2.4. Statistical analysis

All patients included were classified into two groups, clustered and non-clustered. Categorical data were compared by Chi–Square test (or Fisher exact test). *P*-value below 0.05 was considered significant. The minimum estimate of the proportion of tuberculosis caused by transmission was calculated as (Godfrey-Faussett et al., 2000):

$$\frac{\text{Number of clustered patients} - \text{number of clusters}}{\text{Total number of patients}}$$

3. Results

The age of the patients ranged from 12 to 90 years. Seventy (58.3%) of the 120 isolates were susceptible to INH, RIF, SM and ETB. Thirty percent of the isolates were found to be resistant to at least one drug, with 22.5% of the isolates were resistant to STR, 10.83% were resistant to INH, 10% were resistant to RIF and 3.33% were resistant to ETB. Among Iranian isolates only two isolates (1.9%) were MDR and in patients traveled from the Republic of Azerbaijan four isolates (28.6%) were MDR.

Eighty eight distinct profiles were identified, including 20 clustered profiles and 68 unique patterns. Fifty two isolates (43.3%) included in clusters (Table 1). In clusters, the largest cluster comprised six patients. Eight clusters were comprised of three patients and eleven clusters comprised of two patients. In MIRU-VNTR typing three clusters found to share a similar profile with Azeri patients. One Azeri patient who was 35 years old and referred to our center for treatment in several times, showed similar MIRU-VNTR profile with two Iranian patients. The second cluster comprised one Iranian and one Azeri patient who was resistant to SM, and the last cluster included two Azeri patients, which both were resistance to SM and one of them was resistant to INH (Fig. 2). The minimum estimate of the proportion of tuberculosis that was due to transmission in East Azerbaijan province of Iran with MIRU-VNTR was 26.7% [(52–20)/120].

4. Discussion

Treatment of MDR tuberculosis needs long time usage of toxic drugs which is offensive for patients and is costly for patients and governments. It's why identifying recent transmission of MDR tuberculosis is one of concern topics especially in

Table 1 – Traits of clustering tuberculosis in East Azarbaijan Province of Iran. Abbreviations indicates: INH: isoniazid, RF: rifampin, SM: streptomycin, and ETB: ethambutol.

Trait	No. of clustered patients (%)	No. of non-clustered patients (%)	All patients (%)	*P*-value
Resistance to any drug	5 (9.6)	31 (45.6)	36 (30)	$P < 0.01$
Sensitive to Four drugs (INH, RF, SM, ETB)	47 (90.4)	37 (54.4)	84 (70)	$P < 0.01$
Multidrug resistance	1 (1.9)	5 (7.35)	6 (5)	$P = 0.1762$
Non Multidrug resistance	51 (98.1)	63 (92.65)	114 (95)	$P = 0.1762$

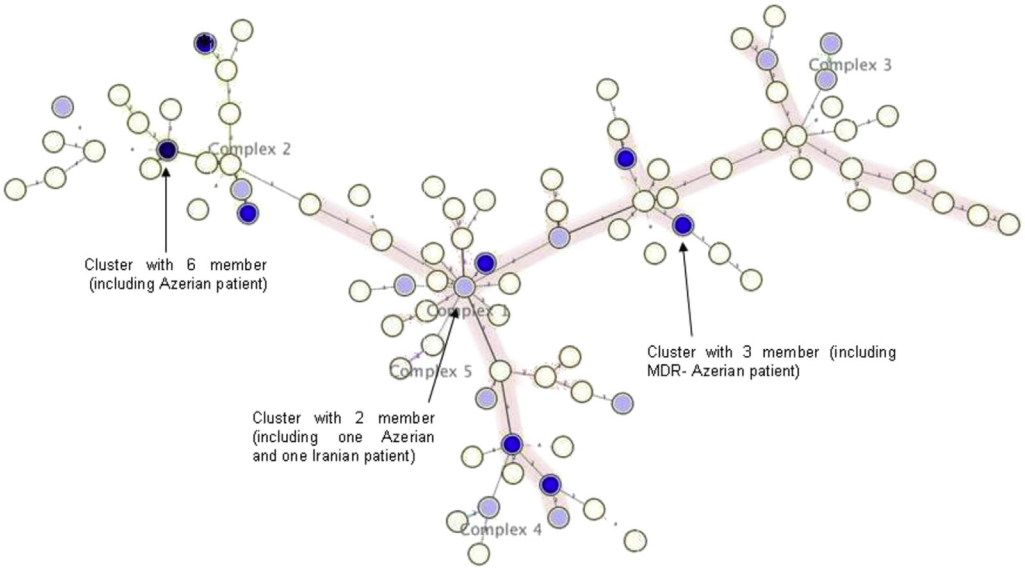

Fig. 2 – Clusters and distribution of isolates in phylogenetic tree. Color circles indicate clusters. Clusters with MDR isolates and Azeri patients are explained in figure.

developing countries. For identifying recent transmission of MDR isolates of *M. tuberculosis*, all clinical isolates should be typed by molecular methods and presence of isolates in the same clusters reveals recent transmissions (van Deutekom et al., 2005). In this study 43.3% of isolates included in clusters which the biggest cluster contained six patients and the rest of clusters included less patients including 2 or 3 patients. This indicates that transmission of MDR tuberculosis is low in East Azerbaijan province, of Iran and there is no sign of transmission of resistant isolates in this region. By hypothesis that all patients included in the same cluster, acquired infections recently from one of cluster members, only 14% of MDR isolates acquired infections recently, whereas it was higher in a study by Frieden and colleagues in New York city (Frieden et al., 1993), they concluded that the prevalence of HIV and Drug abuse by injection caused high rate of MDR transmission in their region. Also in some other regions like Archangel in Russia (Toungoussova et al., 2002), Estonia (Krüüner et al., 2001), South Africa (Marais et al., 2006) transmission rate of MDR tuberculosis were higher than present study. But transmission of MDR-TB in Texas – USA (Wilson et al., 1999) and Los Angles – California (Nitta et al., 2002) they had the same rate of transmission that was due to patient stewardship programs (Nitta et al., 2002). Several factors can contribute in low rate of MDR-TB transmission in our region: 1) Beijing strains are more pathogen (Parwati et al., 2010) and highly transmissible (Toungoussova et al., 2002), most of MDR-TB isolates belonged to this genotype (Marais et al., 2006), considering the fact that there is no Beijing strains report from this province, therefore, it can justify less transmission of MDR-TB in this region. 2) In this study, most of clusters compromised low member and MDR isolates had less tendency to be in clusters in comparing with sensitive isolates. Previously Li et al. showed that Isoniasid resistant isolates were less pathogen in guinea pig in comparing with sensitive isolates and mutation in KatG gene caused resistance to Isoniasid and lower pathogenesis (Li et al., 1998). 3) patients conditions have a direct effect on the emergence of MDR isolates, this condition can be public welfare, prevalence of HIV (Frieden et al., 1993), As reported before, MDR-TB is more prevalent in regions with high prevalence of HIV infected patients (Frieden et al., 1993; Jafari-Khounigh et al., 2014), in this province because of the low prevalence of HIV, we expected less transmission of MDR-TB. 4) This province is a pioneer in TB controlling programs in our country that caused less contamination and transmission of MDR-TB in this province.

This province host patients from several neighbor countries, mostly from Republic of Azerbaijan. In the present study 67% of MDR isolates belonged to Patients from Republic of Azerbaijan which have been referred to the Tabriz TB center for treatment. They had not clear clinical background and at least half of them had resistance to one or more antibiotics and four of them had MDR-TB. Number of patients referred from Rep. of Azerbaijan increased from 12% in 2003 to 22% in 2012. Rate of MDR-TB in East Azarbaijan was about 1.9% while the rate of MDR-TB in isolates from the Rep. Azerbaijan was about 28.6%. Present study showed that rate of TB transmission from Azerian patients to Iranian was 5.8%. Previous study as well as ours has shown 60.8% MDR-TB isolation of Beijing strains from Rep. Azerbaijan (Pfyffer et al., 2001). This high rate of MDR in this country is due to the high cost of TB treatment and weak public health infrastructures, therefore, patients prefers Iranian hospitals and health centers for treatment. According to longtime recurrence nature of TB, and high prevalence of MDR-TB in isolates from Rep. Azerbaijan, we will face with the outbreak of MDR-TB in East Azarbaijan- Iran and we need Comprehensive program to monitor patients and controlling infection transmission.

Results of the present study showed that the isolates of *M. tuberculosis*, which clustered in the same clusters can have different resistance pattern and isolates will get resistance because of inappropriate use of Antibiotics. In one of cluster with three members, one of them was resistant to Streptomycin and Isoniazid but the rest members were only resistant to streptomycin. Also in a cluster with Azeri patient, Azerian isolate was MDR but Iranian isolate was sensitive. This indicates that isolate was sensitive in the first visit of Azerian patients from Iran, but it Acquired resistance during the stay on their country and probably inappropriate usage of antibiotics.

Our limitations in this study included limited MIRU-VNTR typed isolates that contained six month period isolates, that with longer time we will have a clearer assessment of the TB transmission condition in this region. Also we have some limitation in antibiogram performance in our province. Also patient's records were not registered completely like where foreign patients are staying in Iran or with whom they have contact. We hope to solve these obstacles for further studies.

5. Conclusion

In conclusion, results of the present study showed that the high percent of TB cases are sensitive to antibiotics. MIRU-VNTR typing showed a limited number of MDR-TB transmission in our isolates set. Because of the increasing number of patients referred from Rep. of Azerbaijan with MDR-TB, we need more stewardship and controlling these patients for preventing the outbreak of MDR-TB in our region.

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